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MINIREVIEW

Nucleic Acid Amplification Tests for Diagnosis of *Bordetella* Infections

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The global epidemiology of pertussis has recently been reviewed (12, 15). *Bordetella pertussis* continues to circulate even in populations where high vaccination coverage of infants and children is achieved (15, 23), because the protection after natural infection wanes after 10 to 15 years and protection after vaccination lasts for 6 to 10 years (15). A significant increase of pertussis cases was observed in the United States, in Europe, and in other countries with high vaccination coverage, making pertussis a reemerging disease. Transmission of the disease in highly vaccinated populations occurs mainly from adolescents and adults to infants or among older vaccinated children, adolescents, and adults (15). Thus, most cases of pertussis are now observed in unvaccinated infants, older schoolchildren, adolescents, and adults. In outbreak situations asymptomatic carriage has been observed in up to ~50% (12).

CLINICAL PRESENTATION

Clinical symptoms of pertussis are typical in nonvaccinated children and include coughing spasms, whooping, and vomiting. The Centers for Disease Control and Prevention case definition requires 14 days of coughing with paroxysms, whooping, or vomiting for its clinical case definition. However, cases in neonates and unvaccinated infants often present with apnea as the only symptom (15). *B. pertussis* infections in older schoolchildren, adolescents, and adults represent nonprimary contacts with pertussis antigens, and thus the symptoms can vary widely. These are often atypical and may only present as prolonged coughing without other typical findings. Consequently, the clinical diagnosis must be confirmed by laboratory tests. Nonspecific laboratory tests such as differential blood count, erythrocyte sedimentation rate, etc., are mostly not useful in the diagnosis of pertussis. Specific tests are culture, direct immunofluorescence (DFA), PCR, and detection of serum antibodies.

DIAGNOSIS

The pathogenesis of *Bordetella* infections and the application of diagnostic methods have recently been thoroughly reviewed (12) and are only summarized here.

Culture and DFA. Culture is thought to be almost 100% specific, because, so far, in outbreak situations very few patients were found to harbor *B. pertussis* without any symptoms (12).

Sampling for culture is difficult, and it influences the sensitivity markedly. Taking nasopharyngeal aspirates (NPA) from newborns and young infants is a routine procedure in pediatric hospitals, but taking nasopharyngeal swabs from older children, adolescents, and adults requires training, since this swabbing method is not frequently used for other diagnostic purposes in general practice.

The most sensitive method for culture is direct plating and preincubation before transport, which are done mostly in controlled studies. Transport time of aspirates and swabs is critical, and a transport medium (2) protecting the bacteria is required. Transport time should not exceed 48 h.

Culture media, such as Regan-Lowe medium, Bordet-Gengou medium, and Stainer-Scholte medium, have been used for a long time. Incubation time should be at least 1 week, but prolonged incubation periods have been proposed.

Culture varies in sensitivity, being highest for young unvaccinated infants with short duration of symptoms and lowest (<10%) for adolescents and adults with a longer duration of coughing (23). Most countries with statutory notification and laboratory confirmation accept culture as a proof of infection.

DFA also requires nasopharyngeal swabbing. Although rapid and simple, DFA lacks sensitivity and specificity (12). DFA is generally not accepted as a proof of infection in notifying countries.

Serology. Most cases of pertussis in older vaccinated children, in adolescents, and in adults are diagnosed by serological tests outside the United States. The enzyme-linked immunosorbent assay methodology and the type of antigens as well as the reference sera have been standardized, and they have been used in all acellular-vaccine trials, in huge seroepidemiological studies in many countries, and for diagnostic purposes in various laboratories (12). In contrast to this vast amount of data, pertussis serology still suffers from various drawbacks. Pertussis toxin (PT) is often used as the only antigen, but the role of other antigens, such as filamentous hemagglutinin (FHA), pertactin (PRN), fimbriae, adenylate cyclase toxin, and others has not been fully established. Immunoglobulin G (IgG)

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TABLE 1. Proportion of positive samples by real-time *Bordetella* PCR formats compared to culture and block-based PCR formats[a]

n	Sample type	Transport medium	Culture medium	Culture % positive	PCR format	PCR % positive	Reference
148	NPS	Amies charcoal	Regan-Lowe	8	TaqMan IS	30	9
111	NPS	Amies charcoal	Regan-Lowe + DFA	9	LC IS	36	20
					LC PT	10	
100	NPS	Amies charcoal	Regan-Lowe	9	LC IS	21	10
					TaqMan IS	21	
57	NPS, NPA, sputa, throat wash	Stuart's medium/dry	Regan-Lowe	14	TaqMan IS	39	21
					Block-based IS	33	
152	NPS				LC IS	26	1
					Block-based IS	32	
					Seminested IS	37	

[a] Abbreviations: NPS, nasopharyngeal swabs; LC, LightCycler; IS, insertion sequences; PT, pertussis toxin gene.

antibodies are mostly measured, but the role of other isotypes, such as IgA and IgM, is not clear. PT is contained in all vaccines, and FHA, PRN, and fimbriae are components of many acellular pertussis vaccines. Thus, the immune responses against infection and vaccination cannot be distinguished. IgG anti-PT and other antibodies to *Bordetella* antigens are detectable in the majority of all adolescent and adult populations tested so far. Thus, serological diagnosis of pertussis must be done in immunologically nonnaive populations with different kinetics of antibody production. Even in paired sera no antibody increase may be seen after infection, and the diagnosis may also be based on a decrease of antibodies. For reasons of practicability and cost, routine diagnosis must be based on single-sample serology using a single or a continuous cutoff. Irrespective of the huge amount of validation data, no Food and Drug Administration-cleared test for measuring pertussis antibodies is available. Some European countries with statutory notification and laboratory confirmation accept serology as a proof of infection.

Real-time PCR. Due to its sensitivity, specificity, and speed PCR lends itself to the diagnosis of pertussis and it is accepted as a proof of infection in many countries with notification systems. Recommendations for the use of PCR in the diagnosis of *B. pertussis* infections were published previously (13). Since then, real-time PCR formats have emerged and are now widely used. The European Research Programme for Improved Pertussis Strain Characterization and Surveillance (EUpertstrain) held a meeting in September 2004 to revisit the methodology and the application of real-time PCR for detecting *Bordetella* DNA.

Block-based PCR (19) and real-time PCR (Table 1) are more sensitive than culture for the detection of *B. pertussis* and *B. parapertussis*, especially in the late stage of the disease and after antibiotic treatment has been started. Two studies compared the sensitivities of block-based and real-time PCR (1, 20), and the results were equivocal. An external quality control (QC) program found no systematic difference among participants using block-based or real-time PCR (14). Similar to culture, the sensitivity of PCR decreases with the duration of cough; however, due to its higher sensitivity, it may be a useful tool for diagnosis not only for the first 3 to 4 weeks of coughing, but even longer (8). Real-time PCR formats additionally have the advantage of offering a result within several hours.

Sample collection. NPA are the optimal sample for infants; they offer superior sensitivity compared to swabs (7). Nasopharyngeal swabs with Dacron or rayon tips taken by trained personnel provide valid specimens from older children, adolescents, and adults. Calcium alginate swabs should not be used (2). If possible, samples should be taken before antibiotic treatment.

Sputum samples or throat washes may represent an alternative for adolescents and adults. The sensitivity of detecting *Bordetella* DNA in these materials has to be validated because, for culture, throat swabs were found to be less suitable (12).

Sample transport. NPA and dry swabs can be transported at ambient temperature. The use of microbiological transport media, such as Amies medium with charcoal, did not interfere with PCR (10).

Pretreatment of samples. Material from swabs is eluted by soaking the tip for around 30 min in phosphate-buffered saline or lysis buffer depending on the extraction method or commercial kit. A solubilization protocol for respiratory materials can be found in the EUpertstrain website: www.eupertstrain.org.

DNA extraction. Extraction of DNA from the primary specimen is necessary to limit inhibition of PCR (17). Commercially available DNA extraction kits such as the Roche and QIAGEN kits were used by members of this group. Commercially available extraction kits based on silica gel, developed for viral DNA or for total DNA from various sources, were compared in one study. Significant differences in the sensitivity and in the frequency of PCR inhibition were observed (M. Riffelmann et al., submitted for publication). Another study compared alkali lysis and neutralization and a silica adsorption method and found alkali lysis to be more sensitive (K. Gullsby et al., unpublished data).

At this time the Roche High Pure PCR template preparation kit and the QIAGEN QIAamp DNA Mini kit seem comparable and appropriate, but no head-to-head comparison has yet been done. Commercially available kits are not FDA cleared or in conformity with European legal requirements (CE-marked) for this purpose.

Target species/target sequence. Most laboratories use the IS*481* target (11) for the detection of *B. pertussis* and the IS*1001* target (22) for the detection of *B. parapertussis*.

Figure 1 shows the primer-probe combinations in the IS*481* sequence which have been published. U.S. and international

```
  1 GCGAGGCCGGCTATCTGTGAAGATTCAATAGGTTGTATGCATGGTTCATCCGAACCGGAT 60
    ------------------GATTCAATAGGTTGTATGCATGGTT-------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    --------------TCAATAGGTTGTATGCATGG-------------- D

    TTGAGAAACTGGAAATCGCCGACCCCCCAGTTCACTCAAGGAGCCCGGCCGGATGAACAC 120
    --------------TCGCCAACCCCCCAGTTCACTCA--GAGCCCGGCCGGATGAACAC A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    ---------------------------------------GGCCGGATGAACAC D

    CCATAAGCATGCCCGATTGACCTTCCTACGTCGACTCGAAATGGTCCAGCAATTGATCGC 180
    CC---------------------------------------------------CGC A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    CCATAA-------------------------AATGGTCCAGCAATTGATC-- D

    CCATCAAGTTTGTGTGCCTGAAGCGGCCCGCGCCTATGGGGTCACCGCGCCGACTGTGCG 240
    CCATCAAGTTTGTGTGCCTGAA------------------------------ A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    -------------------------------------------------------------- D

    -------------------------------------------------------- 300 - 660

    CATCCCCCCGACGAGCGCTTCCCCAGCGCCGTCCAGTTCCTCAAGGACGCAGTGGCCTA 720
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------CCAGTTCCTCAAGGACGC---------- C
    -------------------------------------------------------------- D

    CTACCAGCGCCTGGGCGTGACCATCCAGCGCGTTGCTCACCGACAATGGCTCGGCCTTTCG 780
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C

    CAGCCGCGCCTTCGCCGCGCTGTGCCATGAGCTGGGCATCAAGCACCGCTTTACCCGACC 840
    -------------------------------------------------------------- A
    -------------------------ATCAAGCACCGCTTTACCC---- B
    ---------------------------CACCGCTTTACCCGACC C
    -------------------------------------------------------------- D

    TTACCGCCCACAGACCAATGGCAAGGCCGAACGCTTCATCCAGTCGGCCTTGCGTGAGTG 900
    -------------------------------------------------------------- A
    --------------------AATGGCAAGGCCGAACGCTTCA---------- B
    TTACCGCCCAC--GACCAATGGCAAGGCCGAACGCTTCAT C
    -------------------------------------------------------------- D

    GGCTTACGCTCACACCTACCAGAACTCCCAACACCGAGCCGATGCCATGAAATCCTGGCT 960
    -------------------------------------------------------------- A
    ---------CACACCTACCAGAACTCCCAA---------- B
    ----TACGCTCACACCTACCAGAACTC---------- C
    -------------------------------------------------------------- D

    -------------------------------------------------------- 1020-1073
```
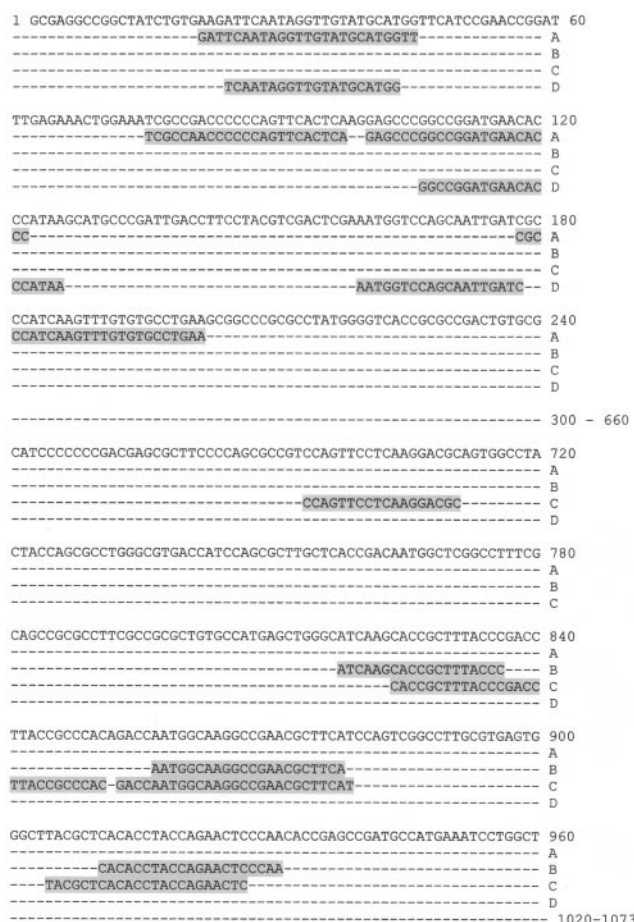
FIG. 1. Location of primer-probe combinations on IS*481*. A, Reischl et al., 2001 (18), and Kösters et al., 2002 (10), FRET-HyProbe; B, Kösters et al., 2001 (9), TaqMan; C, U.S. patent US2003/0165866 (4) and Sloan et al., 2002 (20), FRET-HyProbe; D, Templeton et al., 2003 (21), molecular beacon.

```
  1 ----------------------------------------------------- 360

    GCTTGCCTGGCTGGGGCGATATCAACGGGTGACGGATCGGCTGGCGCAGGCCTGCAGCCA 420
    -------------------------------------------------------------- A
    ---------------GATATCAACGGGTGACGGATC---------- B
    --------------GGCGATATCAACGGGTGA---------- C
    -------------------------------------------------------------- D

    ATTGCTGCAATCGAGCAACGTGCAGGCGGTGGCGAGGTTCTTCGAACTGGGTTGGCATAC 480
    -------------------------------------------------------------- A
    --TGCTGCAATCGAGCAACGTGCA--------------- B
    -----------------TTCGAACTGGGTTGGCATAC C
    -------------------------------------------------------------- D

    CGTCAAGACGCTGGACAAGGCTCGGCTGCGTGCGTCGGTGCGCGAACCGGATTGGTCCAA 540
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -GTCAAGACGCTGGACAAGGCTC---------- C
    -------------------------------------------------------------- D

    GATCGAGTATTTGGCGATGGACGAGTTTGCCCTGCACAAAGGGCATCGCTACGCGACAGT 600
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    --------------GATGGACGAGTTTGCCCTG---------- C
    -------------------------------------------------------------- D

    ----------------------------------------------------- 660-720

    CATCGACATGACCACCGCCTACGAGTTGGAGATCCAGGCCCACAGCCCACAGGCGGAGAT 780
    ---------------CACCGCCTACGAGTTGGAGAT---------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    -------------------------------------------------------------- D

    CGTCTATGACTTGTTCCATGTCGTGGCCAAGTATGGACGAGAGGTCATTGATCGGGTGCG 840-900
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    --------------CCATGTCGTGGCCAAGTA---------- D

    TCGCTGGCTGCTGCTGCGCAACCGTGACAACCTGGATCGGCAGCAGGCCGTCCGGCTCGA 960
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    --GCTGGCTRCTGCTGCGCAA---------- D

    CGAATTGCTGCAAGCCAACCAGCCGCTGCTGACGGTCTATGTCCTGCGTGACGAACTCAA 1020
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    ----TTGCTGCAAGCCAACCA---------- D

    ACGGCTCTGGTTCTACCAAAGACCTGCCTGGGCAAGACAAGCCTGGAACCACTGGTACGA 1080
    --------GTTCTACCAAAGACCTGCCTGGGC-AGACAAGCCTGGAACCACTGGTAC-- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    -------------------------------------------------------------- D

    GCAGGCCGAGCAAAGCGGAATAGCCGCCTTGAACACCTTCGCTCAGCGCTTGAAAGGCTA 1140
    -------------------------------------------------------------- A
    -------------------------------------------------------------- B
    -------------------------------------------------------------- C
    -------------------------------------------------------------- D

    TCTGCACGGCATCCTGGCCAGATGCCGACATCCCCTGAACACCAGCATTGTCGAGGGCAT 1200
    -------------------------------------------------------------- A
    ---------------TGAACACCAGCATTGTCGAGG---- B
    -------------------------------------------------------------- C
    -------------------------------------------------------------- D

    ----------------------------------------------------- 1306
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FIG. 2. Location of primer-probe combinations on IS*1001*. A, Reischl et al. 2001 (18), and Kösters et al. 2002 (10), FRET-HyProbe; B, Kösters et al. 2001 (9), TaqMan; C, U.S. patent US2003/0165866 (4) and Sloan et al., 2002 (20), FRET-HyProbe; D, Templeton et al. 2003 (21), molecular beacon.

patents have been granted for primer-probe sets in IS*481* (4, 12a). There has been concern about the specificity of detection of *B. pertussis* due to sequence identity with *B. holmesii* (11, 18). To address this, more than 1,000 clinical samples were retested using primer-probe sets specific for *B. holmesii* DNA (A. van der Zee et al., personal communication). Additionally, a real-time PCR protocol for *recA* of *B. holmesii* was developed, and more than 220 clinical samples positive by IS*481* PCR were retested (M. Antila et al., submitted for publication). *B. holmesii*-specific sequences were not detected in any of the retested samples. These results suggest that IS*481* assays may currently be sufficiently specific for the laboratory diagnosis of *B. pertussis*. However, the apparent periodic appearance of *B. holmesii* in some host populations, together with the possible carriage of IS*481* by some strains of *B. parapertussis*, *B. bronchiseptica*, *B. avium*, and *B. petrii* (V. Caro et al., unpublished data), makes monitoring the specificity of IS*481* necessary.

No specificity problems were reported for the detection of IS*1001* to diagnose *B. parapertussis* infections, although *B. holmesii* 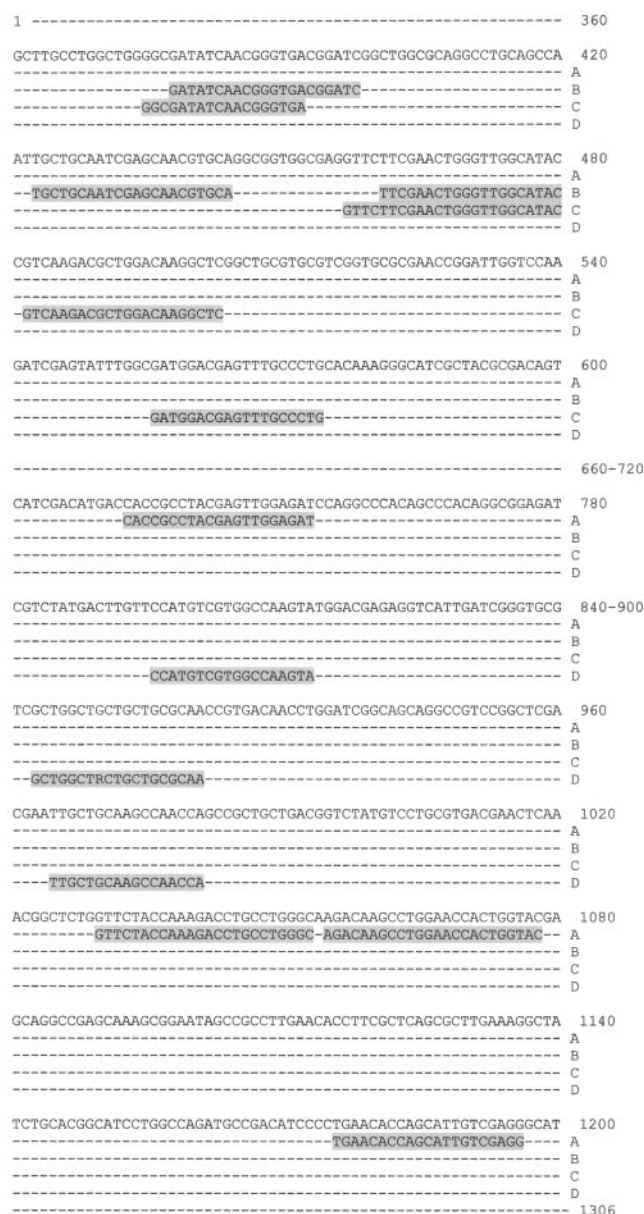shares some sequence identity (21). This insertion sequence seems a suitable target for human as well as for ovine isolates (Caro et al., unpublished). Figure 2 shows the primer-probe combinations in the IS*1001* sequence that have been published or patented.

Although the pertussis toxin operon is present in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, the pertussis toxin promoter (*ptxA*-Pr) is a target for *B. pertussis*-specific assays using a real-time format (5, 14). It was, however, consistently less sensitive than IS*481* (Caro et al., unpublished). Some reference laboratories are using both targets (5, 6), which is costly. A real-time PCR format on a LightCycler using the pertussis

toxin gene (not the promoter) has been published (20), which will detect *B. pertussis* and *B. parapertussis*.

Real-time formats. Sequence-specific detection can be done by fluorescence resonance energy transfer (FRET) hybridization probes (10, 18, 20), TaqMan probes (9), and molecular beacons (21). A non-sequence-specific format using SYBR green I has also been described (16). The consensus group agreed that detection of amplicons should be done by sequence-specific formats.

Most protocols use simplex PCR for either *B. pertussis* (*B. holmesii*) or *B. parapertussis*. A duplex PCR for *B. pertussis* and *B. parapertussis* showed a significant loss in sensitivity for clinical samples compared to the simplex PCR (10).

Prevention of crossover contamination. All real-time PCR formats minimize crossover contamination by design. Using the uracil-DNA glycosylase system, additional crossover contamination prevention is possible, and some commercially available generic reaction mixtures contain the basic reagents (i.e., LightCycler FastStart DNA master hybridization probes; Roche).

Run controls and standardization. (i) Extraction controls. Internal nucleic acid extraction controls can be done by spiking the samples with an internal process control (see below). Bacterial lysis cannot be internally controlled for every sample; thus a positive control (*Bordetella* bacterial suspensions) should be included in every run. At least one negative control (mostly PCR grade water) is necessary to exclude contamination of the system.

(ii) Internal amplification control. For real-time PCR formats, an internal amplification control seems necessary. Internal controls based on the separate amplification of a phocine herpesvirus (21) or on a system competitive to the PT promoter PCR (6) or to the IS*481* PCR (3) have been described. Almost all samples tested so far harbored human gene sequences, and thus their amplification can also be used as an inhibition control.

(iii) Positive and negative in-run controls. In-run controls should include negative controls (PCR grade water) and positive controls. Typically, nonstandardized purified DNA (controlling for amplification and extraction) or a bacterial suspension (also controlling for bacterial lysis) are used as positive controls.

A standardized total DNA extract from a sequenced strain, *B. pertussis* Tohama I or *B. parapertussis* ATCC 12822, would be desirable.

External quality control. External quality programs have been implemented in some European countries (14). An effort is ongoing with the Quality Control of Molecular Diagnostics organization (Glasgow, Scotland) to offer external QC on a European and international scale.

Results. (i) Clinical samples. For practical purposes, a positive IS*481* PCR can be considered to be a *B. pertussis* infection, when the clinical symptoms are in accordance with this result. The specificity may be further substantiated by a positive *ptxA*-Pr PCR by reference laboratories. A positive IS*1001* PCR result is considered to be indicative of *B. parapertussis* infection.

The sensitivity of PCR appears to be highest in newborns and young infants (5, 6). In adolescents and adults, the sensitivity depends on the time between cough onset and specimen collection and on the bacterial load but was found to be always lower than 50% (23).

For interpreting all *Bordetella* real-time PCR results the positive predictive values require more evaluation of various factors such as age, disease severity, and vaccination status. It is important to keep in mind that a positive PCR may be found in outbreak situations and after household contact in patients with very little or no symptoms (12).

(ii) Epidemiological studies. Positive results from an IS*481* real-time PCR should be regarded as evidence for infection with *Bordetella* spp. The specificity of the result must be substantiated by another PCR with a *B. pertussis*-specific target. It has to be noted that the positive detection limit of IS*481* was defined as 10 fg per reaction (Caro et al. and N. K. Fry et al., unpublished data).

A positive IS*1001* PCR result can be considered evidence for *B. parapertussis* infection. From earlier data it is assumed that no relevant numbers of wholly asymptomatic carriers will be detected, but, when real-time PCR formats are used, this assumption has to be validated by transmission studies.

CONCLUSIONS AND PERSPECTIVES

Real-time PCR formats have increased the speed and sensitivity of diagnosis compared to culture, although all procedures need more standardization and optimization. Recommendations include extraction controls, internal controls, a *B. pertussis* and *B. parapertussis* DNA standard, and participation in an external QC program. For epidemiology and surveillance purposes, the role of *B. holmesii* and of other *Bordetella* species may require a genus-specific real-time PCR format, followed by a species-specific format. So far, no published procedure has comprised all necessary controls including those for consistency of bacterial lysis and yield of DNA extraction. Commercially available kits with appropriate validation would significantly facilitate the work of medical laboratories, and such CE-marked kits are a requirement in the European Union as from 2005. Further developments in automation of sample preparation and PCR procedures are ongoing, and *Bordetella* PCR will be just one of many applications to be run on mechanized PCR systems.

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